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PU.1 and Spi-B Are Required for Normal B Cell Receptor–Mediated Signal Transduction

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Summary

PU.1 and Spi-B have previously been implicated in the regulation of genes encoding B cell receptor (BCR) signaling components. *Spi-B*$^{-/-}$ B lymphocytes respond poorly to BCR stimulation; *PU.1*$^{-/-}$ mice, however, lack B cells, precluding an analysis of BCR responses. We now show that *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells exhibit more extensive defects than *Spi-B*$^{-/-}$ B cells, indicating that both PU.1 and Spi-B are required for normal BCR signaling. Strikingly, BCR cross-linking results in substantially reduced protein tyrosine phosphorylation in mutant B cells. Further analysis shows that Igα is phosphorylated and syk is recruited and becomes phosphorylated but that BLNK and PLCγ phosphorylation are defective in mutant cells. Our data support the existence of a novel component coupling syk to downstream targets.

Introduction

The B cell receptor (BCR) complex consists of membrane-bound immunoglobulin (Ig) noncovalently associated with two signal-transducing proteins, Igα (CD79a) and Igβ (CD79b) (reviewed in Cambier et al., 1994). BCR signaling is critically important at many stages of B cell development. For example, during the early stages of B lymphopoiesis in the bone marrow, signals transduced through a pre-BCR complex are necessary for positive selection of cells expressing a functional Ig heavy chain allele, for allelic exclusion of further heavy chain rearrangements, and for the expansion of precursor populations (Kitamura et al., 1991, 1992; Gong and Nussenzweig, 1996). Immature B cells in the bone marrow and peripheral tissues whose BCR molecules are reactive with self-antigen undergo receptor editing, deletion, or inactivation (anergy). Mature B cells in the periphery require the presence of surface BCRs and presumably low levels of BCR signaling for survival (Lam et al., 1997). Moreover, recognition and internalization of foreign antigens by B cells occurs via their binding to the BCR complex. Finally, during the process of affinity maturation, interaction of antigens with the BCR complex is necessary to select high-affinity antibodies. Therefore, study of the BCR signaling cascade and factors that regulate it is critical to understanding B cell development and function.

The complexity of BCR function may reflect its interaction with multiple signal transduction pathways. For example, binding of antigens to cell surface Ig results in rapid phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of Igα and Igβ (Gold et al., 1991). Members of the src kinase family (lyn, fyn, blk, etc.) are associated at low stoichiometry with the unstimulated BCR complex (Lin and Justement, 1992) and have been implicated in phosphorylating Igα and Igβ as well as numerous other cellular substrates upon BCR stimulation. The tyrosine kinase syk is thought to be recruited to phosphorylated Igα and Igβ cytoplasmic tails via its tandem SH2 domains and has been shown to phosphorylate additional signaling molecules, including phospholipase C (PLC) (Law et al., 1996a).

In addition to kinases, several phosphatases (SHP-1, SHIP-1, and CD45) and adaptor proteins (BLNK/SLP-65, Grb2, Shc, and Nck) also regulate BCR signal transduction (Justement et al., 1991; Park and Rhee, 1992; Lankester et al., 1994; Smit et al., 1994; Campbell and Klinman, 1995; Law et al., 1996b; Pao et al., 1997; Bolland et al., 1998; Fu et al., 1998; Wienands et al., 1998). The protein BLNK/SLP-65, which shares homology with the T cell adaptor protein SLP-76, appears to serve as a scaffolding protein mediating interaction of syk with PLCγ, Vav, Grb2, and Nck (Fu et al., 1998; Wienands et al., 1998). BCR stimulation induces protein tyrosine phosphorylation, the mobilization of Ca^{2+} stores, and protein kinase C and MAP kinase activation (reviewed in Tamir and Cambier, 1998), resulting in B cell functional responses including proliferation, isotype switching, and/or antibody secretion.

Binding sites for the Ets family of transcription factors have been identified in several genes encoding BCR signaling proteins including Ig heavy and light chains (Pongubala et al., 1992; Eisenbeis et al., 1993; Nelsen et al., 1993), Ig J chain (Shin and Koshland, 1993), Igα (Feldhaus et al., 1992; Hagman and Grosschedl, 1992), Igβ (Omori and Wall, 1993), and the tyrosine kinases Btk (Sideras et al., 1994; Muller et al., 1996) and blk (Lin et al., 1995). Although seven Ets family members are expressed in B cells (Ets-1, Ets-2, PU.1, Spi-B, Fli-1, Elf-1, and GABPα) (Bassuk and Leiden, 1997), two of these proteins, PU.1 and Spi-B, are particularly abundant. PU.1 and Spi-B are closely homologous to each other but less well conserved with other Ets proteins (Ray et al., 1992). Spi-B is expressed exclusively in lymphoid cells: high levels are detected in B cells and low levels in T cell progenitors (Ray et al., 1992; Chen et al., 1995a; Su et al., 1996). In contrast, expression of PU.1 is more widespread in hematopoietic cells, including both myeloid (granulocyte, monocyte, and osteoclast) and lymphoid (B and immature T lymphocytes) lineages and also hematopoietic progenitor cells (Klemsz et al., 1990; Ray et al., 1992; Voso et al., 1994; Chen et al.,

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1995a; Su et al., 1996; Tondravi et al., 1997). In vitro assays have shown that Spi-B and PU.1 bind to similar or identical DNA sequences and transactivate the same reporter gene constructs in transient transfection assays (Ray et al., 1992; Chen et al., 1995b; Muller et al., 1996; Su et al., 1996). Because PU.1 and Spi-B are expressed in overlapping patterns in mice and bind similar DNA sequences, it is likely that they regulate overlapping sets of target genes and may be in part functionally redundant.

To determine the in vivo function of PU.1 and Spi-B, we have disrupted the genes encoding these proteins in "knockout" mice. Deletion of *PU.1* results in a complete block to lymphoid (B and T cells) and myeloid (macrophages and neutrophils) development during fetal hematopoiesis and in late-stage embryonic lethality (Scott et al., 1994). More recent data indicate that PU.1 is required for the survival and differentiation of a previously unknown lymphoid-myeloid progenitor (Scott et al., 1997). In contrast, independently generated *PU.1*$^{-/-}$ mice survive embryogenesis and birth but subsequently die from pathogenic infection (McKercher et al., 1996). Neonatal mice with this *PU.1* allele develop some B220^{+} cells in bone marrow and spleen, but these cells fail to differentiate properly or to rearrange the Ig heavy chain. Since both *PU.1* alleles result in a lack of mature B cells, it has not previously been possible to define a role, if any, for PU.1 in B cells.

Surprisingly, mutation of the *Spi-B* locus results in viable mice that do not display detectable developmental defects in any hematopoietic lineages (Su et al., 1997). However, *Spi-B*$^{-/-}$ B cells exhibit a functional defect in their ability to respond to antigenic stimulation through the BCR. *Spi-B*$^{-/-}$ mice produce reduced amounts of antibody in response to immunization with a T-dependent antigen, dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH). Examination of spleens of immunized animals shows that *Spi-B*$^{-/-}$ mice develop small, ill-defined germinal centers compared to wild-type mice. Moreover, these germinal centers deteriorate early due to increased B cell apoptosis. In vitro, *Spi-B*$^{-/-}$ B cells proliferate poorly in response to BCR stimulation, whereas proliferation in response to lipopolysaccharide (LPS) is normal. Together, these results suggest that *Spi-B*$^{-/-}$ B cells are defective in one or more components of the signaling pathways activated by BCR stimulation. Moreover, these data indicate that Spi-B performs some unique function(s) in B cells not compensated by the closely related PU.1 protein.

Collectively, these studies indicate that both Spi-B and PU.1 are important in hematopoiesis but play distinct roles. As they bind identical DNA elements, however, PU.1 and Spi-B may also share some overlapping functions masked in the respective "knockout" animals due to functional redundancy. To identify possible roles of PU.1 in B lymphocytes and assess redundancy with Spi-B, we crossed *PU.1*$^{+/-}$ mice to *Spi-B*$^{-/-}$ mice to generate animals with the genotypes *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$, and *PU.1*$^{-/-}$*Spi-B*$^{-/-}$. We show that both PU.1 and Spi-B are required for normal BCR signal transduction in that mutant B cells exhibit greatly reduced tyrosine phosphorylation and Ca^{2+} mobilization upon BCR cross-linking. Our data indicate that syk is recruited to Igα/Igβ and phosphorylated upon BCR cross-linking but that downstream targets are not properly phosphorylated. These results suggest the existence of a previously unknown component of the BCR signaling cascade coupling syk to downstream effectors whose expression is regulated by PU.1 and Spi-B. In keeping with the BCR signaling defect, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice exhibit increased rates of B cell apoptosis and reduced numbers of peripheral B cells. Surprisingly, despite the severe BCR signaling defect in mutant animals, B cell lymphopoiesis is apparently normal, raising the possibility that signal transduction through the pre-BCR and BCR complexes is not identical.

Results

Generation of *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ Mice

To investigate the possibility of functional redundancy between PU.1 and Spi-B and the role, if any, of PU.1 in B cells, we crossed *PU.1*$^{+/-}$ and *Spi-B*$^{-/-}$ mice to generate animals with the genotypes *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$, and *PU.1*$^{-/-}$*Spi-B*$^{-/-}$. Although *PU.1*$^{-/-}$ mice fail to generate any myeloid or lymphoid cells and die in the latter half of embryogenesis, it was important to determine whether *PU.1*$^{-/-}$*Spi-B*$^{-/-}$ mice exhibited a more severe defect indicating a possible role for Spi-B in hematopoietic progenitors. However, *PU.1*$^{-/-}$*Spi-B*$^{-/-}$ and *PU.1*$^{-/-}$ embryos showed identical flow cytometry profiles for all hematopoietic lineages at every time point examined (data not shown). Moreover, *PU.1*$^{-/-}$ and *PU.1*$^{-/-}$*Spi-B*$^{-/-}$ embryos died at the same stage of embryonic development. Therefore, Spi-B does not appear to be necessary for the development of hematopoietic progenitors.

In contrast, mice with the genotypes *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ were viable and fertile, allowing us to examine adult hematopoiesis and blood cell function. Both PU.1 and Spi-B have been shown to transcriptionally activate the *PU.1* gene (Chen et al., 1995b), indicating the possibility that B cells from mutant animals might express lower levels of PU.1 protein than expected based on their genotype. PU.1 protein levels in mutant B cells were examined via Western blot analysis using extracts of B cells purified from the spleens of adult mice. PU.1 expression levels correlated with the *PU.1* genotype (i.e., *PU.1*$^{+/-}$ cells express approximately half as much PU.1 protein as do *PU.1*$^{+/+}$ cells) irrespective of the *Spi-B* genotype (data not shown).

Examination of Hematopoietic Lineages in Mutant Mice

We examined the presence of various hematopoietic lineages in *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice compared to wild-type and *Spi-B*$^{-/-}$ mice using flow cytometric analysis of characteristic cell surface antigens. Flow cytometric profiles for myeloid lineages were identical in all genotypes (data not shown). Since both PU.1 and Spi-B are expressed in T cell progenitors and then downregulated as the cells mature, T cell development might be compromised in mutant animals. However, CD4/CD8 profiles in thymus and spleen and TCR$\alpha\beta$/CD3 profiles in the spleen of adult animals were comparable (data not shown), suggesting that the development and survival of T cells in adult mice is normal.

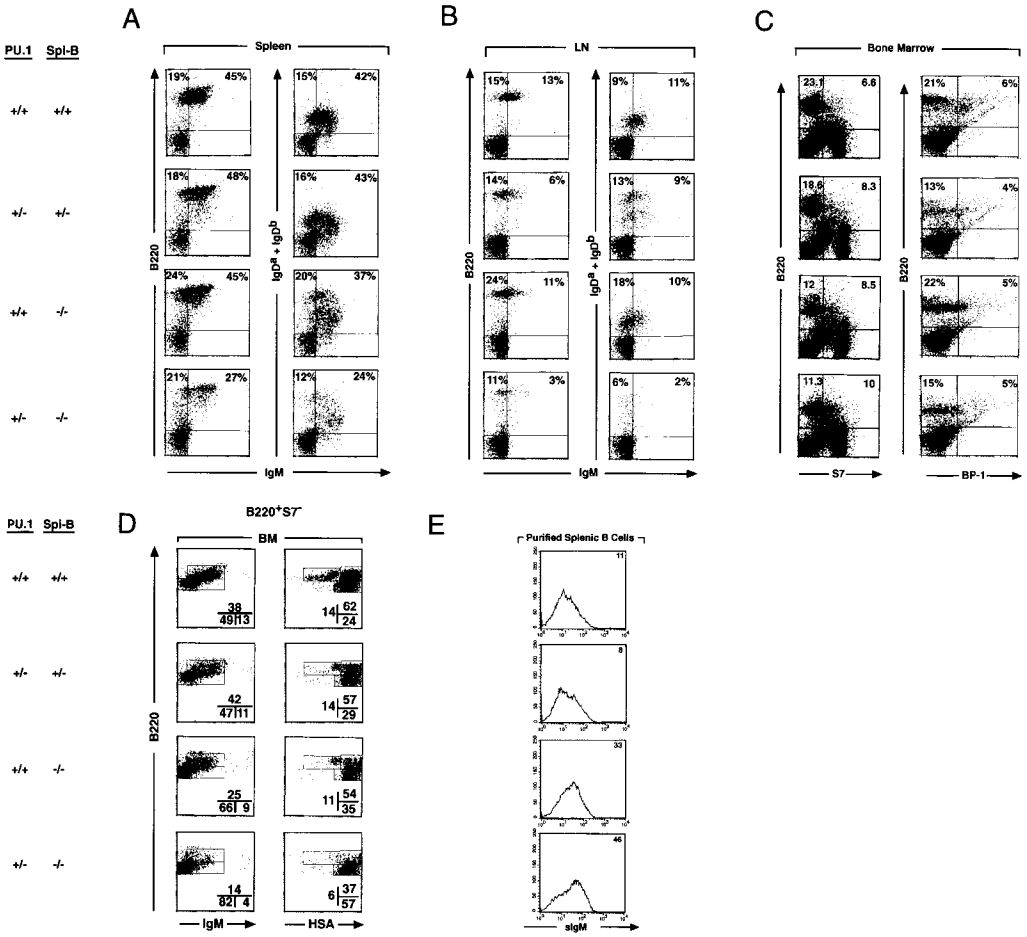

Figure 1. A B Cell Deficiency Is Present in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ Mice

(A and B) *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice exhibit fewer immature and mature B lymphocytes in the spleen and lymph nodes as compared to wild-type littermates (absolute numbers of B cells in spleen: wild-type $31.1 \times 10^6 \pm 1.7 \times 10^6$, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ $15.9 \times 10^6 \pm 3.3 \times 10^6$ [$p < 0.02$], and lymph node: wild-type $2.8 \times 10^6 \pm 0.5 \times 10^6$, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ $1.0 \times 10^6 \pm 0.1 \times 10^6$ [$p < 0.07$]). Flow cytometry performed on the indicated tissue specimens assayed B220/IgM and IgD/IgM staining. Differences in IgD staining between genotypes are largely due to differences in fluorescence intensity between the IgD^a and IgD^b antibodies used. Modest reductions in the numbers of IgM$^+$IgD$^-$ and IgM$^+$IgD$^+$ B cells were detected in 30% of the *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ animals. (C) *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice produce normal numbers of pro-B and pre-B cells. FACs analysis of total bone marrow cells showed that the number of B220$^+$S7$^+$ (pro-B) cells in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice was normal, whereas the number of B220$^+$S7$^-$ (comprising to the pre-B, immature B, and mature recirculating B) cells was reduced. To further define the developmental stage at which differences were noted, B220/BP-1 staining was performed. *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice showed normal numbers of B220$^+$BP-1$^+$ (pro- and pre-B) cells but reduced numbers of B220$^+$BP-1$^-$ (immature and mature recirculating B) cells. Differences in the total number of B220$^+$ cells between panels is due to the fact that different mice were used in the two experiments. To confirm stage at which B cell numbers were reduced, three-color FACs analyses were performed. The B220$^+$S7$^-$ population was gated and is shown in (D). The majority of the B220$^+$S7$^-$ cells in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ bone marrow are B220^{int}IgM$^-$ and B220^{int}HSA^{hi}, signifying the presence of pre-B cells at the expense of more differentiated cell types. B220^{hi}IgM$^+$HSA^{lo} immature and mature B cells represent a minor population in the *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ bone marrow. These results are consistent with the presence of normal numbers of B220$^+$BP-1$^+$ pro- and pre-B cells in mutant bone marrow, shown in (D). (E) Mutant B cells express increased levels of surface IgM. Purified splenic B cells from *PU.1*$^{+/+}$*Spi-B*$^{+/+}$, *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, *PU.1*$^{+/+}$*Spi-B*$^{-/-}$, and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice were stained with IgM-FITC and analyzed by flow cytometry. The peak fluorescence intensity for each sample (indicated by the numbers in the upper right corner) shows that B cells from *PU.1*$^{+/+}$*Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice on average express higher levels of surface IgM than B cells from *PU.1*$^{+/+}$*Spi-B*$^{+/+}$ or *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ mice.

PU.1 and Spi-B are both abundantly expressed in B cells and their progenitors. Flow cytometric analysis (n = 6 for each genotype) showed that *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice exhibited a reduced number of B220$^+$IgM$^+$ and IgM$^+$IgD$^+$ B cells in the spleen (50% reduction) and lymph nodes (60% to 80% reduction) (Figures 1A and 1B), whereas *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ mice were normal. Furthermore, the reduced percentage of B cells in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice was correlated with a reduced absolute number of B cells. We noted that surface IgM was higher in splenocytes of mutant animals than wild-type littermates. Figure 1E shows that B cells purified from *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ spleens display 2- to 4-fold higher levels of surface IgM staining. Differences in IgD staining noted in Figures 1A and 1B are due to differences in the fluorescent intensity of the antibodies for IgD^a and IgD^b (our mice are hybrids of C57BL/6 and 129Sv and express either IgD^a, IgD^b, or both). However, we cannot rule out that the increased levels of IgD staining noted in *Spi-B*$^{-/-}$, *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, and

PU.1$^{+/-}$*Spi-B*$^{-/-}$ mice may be in part due to increased surface IgD levels similar to the results obtained with surface IgM. Mutant B cells also express higher levels of surface CD19 and CD21 than do wild-type cells (data not shown).

To determine the stage of B cell development with a reduction in cell numbers, we examined *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ bone marrow. We detected 50% fewer IgM$^+$IgD$^-$ and IgM$^+$ IgD$^+$ B cells in the bone marrow of *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice (data not shown). Subsequent analysis showed that the percentages of B220$^+$S7$^-$ and B220$^+$BP-1$^-$ B cells in bone marrow were reduced whereas the percentage of B220$^+$S7$^+$ and B220$^+$BP-1$^+$ cells was normal (Figure 1C). To further define the developmental stage at which B cell numbers were reduced, three-color flow cytometry of bone marrow suspensions was performed (Figure 1D), which indicated that the B220$^{hi/int}$S7$^-$IgM$^+$ (immature and mature B cells) population was reduced whereas the B220^{int}S7$^-$IgM$^-$ population (pro- and pre-B cell) was normal (Hardy et al., 1991). Confirming these results, we noted that the B220^{hi}S7$^-$HSA^{lo} population was reduced whereas the B220^{int}S7$^-$HSA^{hi} population was normal (Figure 1D). Therefore, adult *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice have normal numbers of pro- and pre-B cells in the bone marrow but show a deficiency in the numbers of immature and mature B cells in the bone marrow and peripheral tissues. Interestingly, examination of B cell precursors in the fetal liver at embryonic day 16.5 showed normal numbers of B220$^+$S7$^+$ cells but reduced numbers of B220$^+$S7$^-$ cells, indicating that a B cell deficiency is present in fetal as well as adult hematopoiesis (data not shown).

The Ig kappa (κ) and lambda (λ) light chain genes are putative targets of Ets transcription factors (Pongubala et al., 1992; Eisenbeis et al., 1993), and mice that lack κ light chain expression exhibit a 50% reduction in the numbers of peripheral B cells (Chen et al., 1993) similar to the B cell deficiency noted in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice. Flow cytometric analysis of B cell populations in the bone marrow and periphery detected normal levels of cell surface κ and λ on mutant B cells, demonstrating that both of these gene products are expressed normally (data not shown). Moreover, expression of the κ light chain gene in sorted pre-B (B220$^+$ S7$^-$IgM$^-$) cells isolated from bone marrow of *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice was normal based on RNase protection assays of κ constant region transcription (data not shown). Therefore, we have excluded reduced expression of Ig light chain genes as a cause of B cell deficiency in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice.

Flow cytometric analysis of bone marrow populations in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice showed normal numbers of pro-B and pre-B cells indicating that there is no developmental block to B lymphopoiesis. However, we detected reduced numbers of immature and mature B cells in these mice, implying that these cells may be lost at increased rates in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ animals. To test this hypothesis, we examined the rate of apoptosis of IgM$^+$IgD$^-$ immature and IgM$^+$IgD$^+$ mature B cells in spleen (Figure 2) using Annexin V staining. Three to four times as many Annexin V^{hi} apoptotic immature and mature B cells were detected in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice than in mice with other genotypes (n = 3). Furthermore, four times as many B220$^+$ TUNEL$^+$ lymphocytes were present in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ spleens as those harvested from wild-type animals (data not shown). Therefore, increased apoptosis of immature and mature B cells in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice probably accounts for the reduced number of B cells in the bone marrow and peripheral lymphoid organs.

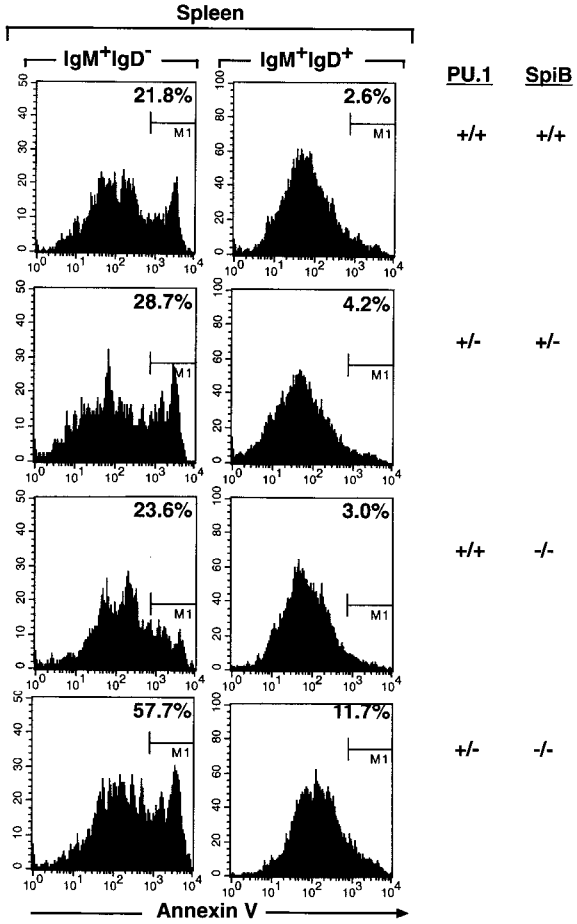

Figure 2. IgM$^+$IgD$^-$ and IgM$^+$IgD$^+$ B Lymphocytes in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ Spleen Exhibit High Levels of Apoptosis

Spleen cells were stained with IgM (cychrome) and IgD (PE), incubated with Annexin V (FITC), and analyzed by flow cytometry. IgM$^+$IgD$^-$ and IgM$^+$IgD$^+$ populations were gated. Increased rates of apoptosis were observed for both immature and mature B cells in spleens obtained from *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ animals.

Mutant Animals Exhibit B Cell Functional Defects

We have previously shown that *Spi-B*$^{-/-}$ mice, upon immunization with a T-dependent antigen DNP-KLH, form small, poorly organized germinal centers that deteriorate early (Su et al., 1997). The mutant germinal centers are characterized by increased B cell apoptosis, likely accounting for their premature disappearance. We asked whether *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice showed similar or more severe defects in germinal center formation. Peanut agglutinin (PNA) staining of splenic sections on days 10 and 28 post antigenic challenge with DNP-KLH showed clearly defined germinal centers in wild-type mice (Figure 3). Both *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ mice developed smaller germinal centers at day 10 that had disappeared by day 28. In marked contrast, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice showed no evidence of

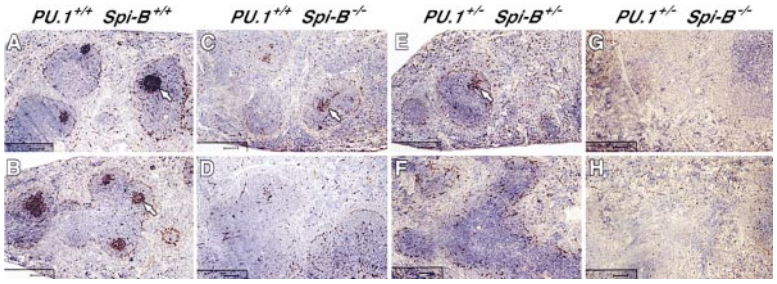

Figure 3. *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ Mice Fail to Form Germinal Centers

Spleen sections were obtained from *PU.1*$^{+/+}$*Spi-B*$^{+/+}$ (A and B), *PU.1*$^{+/+}$*Spi-B*$^{-/-}$ (C and D), *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ (E and F), and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice (G and H) at day 10 (A, C, E, and G) and day 28 (B, D, F, and H) post DNP-KLH immunization and stained with peanut agglutinin (PNA). PNA^{+} germinal center B cells appear as dark brown areas as indicated by the white arrows.

PNA^{+} germinal centers at either day 10 or day 28 post immunization.

We have shown that early deterioration of germinal centers in *Spi-B*$^{-/-}$ mice is correlated with an increased number of B220^{+}/TUNEL^{+} cells in immunized mice than in wild-type animals (Su et al., 1997). To determine whether the lack of germinal centers in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice was also consistent with increased rates of apoptosis of splenic B cells, we performed B220/TUNEL staining. On day 21 post DNP-KLH immunization, *PU.1*$^{+/-}$ *Spi-B*$^{-/-}$ spleens contained 3–4 times as many apoptotic cells as wild-type spleens. These apoptotic cells were largely B cells as assessed by costaining with an anti-B220 antibody. Therefore, like *Spi-B*$^{-/-}$ mice, *PU.1*$^{+/-}$ *Spi-B*$^{-/-}$ mice exhibit increased rates of B cell apoptosis in the spleen upon immunization with a T-dependent antigen. This increased susceptibility of B cells to apoptosis may explain the failure of *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice to form germinal centers. We conclude that *Spi-B*$^{-/-}$, *PU.1*$^{+/-}$ *Spi-B*$^{+/-}$, and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice exhibit defects in germinal center formation and maintenance accompanied by increased rates of apoptosis.

To further examine the functional response of mutant B cells, we purified B cells from the spleens of wild-type and mutant mice and studied their in vitro proliferative responses to various mitogenic stimuli. Like *Spi-B*$^{-/-}$ B cells, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells showed a defect in their proliferative response to an anti-IgM antibody (Figures 4A and 4B), but the defect was significantly more severe (10- to 20-fold reduction [$p < 2 \times 10^{-6}$] versus a 3-fold reduction for *Spi-B*$^{-/-}$ or *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ ($p < 2 \times 10^{-3}$). Mutant B cells proliferated normally in response to an anti-CD40 antibody and the cytokine IL-4; however, these costimulatory signals were not sufficient to rescue poor proliferation in response to BCR ligation (Figure 4B).

Proliferation in response to the polyclonal B cell mitogen LPS is nearly normal for *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$ *Spi-B*$^{+/-}$ B cells, whereas *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells show a 2- to 3-fold reduction in their response to this compound ($p < 0.003$) (Figure 4A). Importantly, B cells of all genotypes were capable of normal proliferation in response to phorbol 12-myrisate 13-acetate (PMA, an activator of protein kinase C [PKC]) and ionomycin (a Ca^{2+} ionophore) (Figure 4A). PMA and ionomycin mimic the signaling pathways downstream of the BCR in part by activating the same pathways as phospholipase C (PLC). Since mutant B cells respond normally when stimulated by PMA and ionomycin but fail to respond properly to BCR stimulation, we conclude that the defect in these cells lies in a membrane-proximal step between the BCR and PLC (see Figure 6).

Mutant B Cells Exhibit Defective Tyrosine Phosphorylation and Ca^{2+} Mobilization upon BCR Cross-Linking

Reduced in vitro proliferation potential to IgM cross-linking indicates that *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells exhibit abnormal responses to BCR-mediated

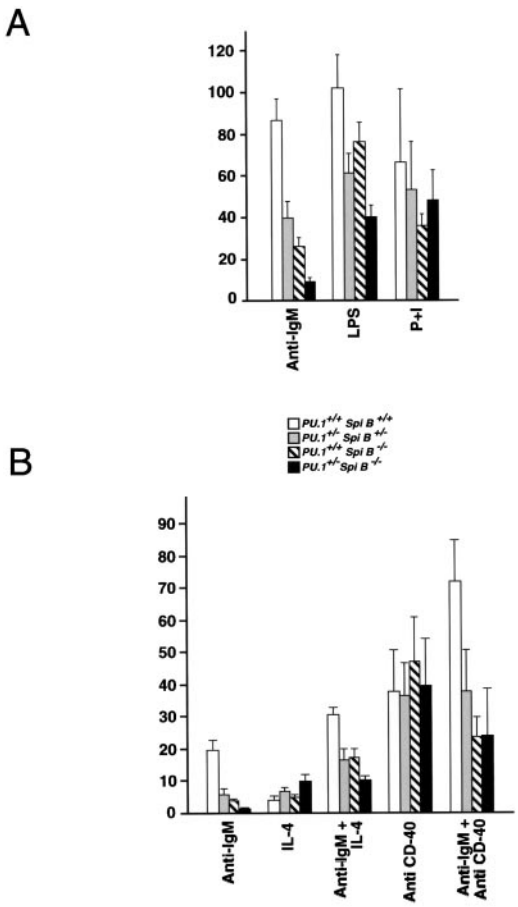

Figure 4. B Cell Proliferative Responses Assayed In Vitro

Splenic B cells were isolated from *PU.1*$^{+/+}$*Spi-B*$^{+/+}$ (white bars), *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ (gray bars), *PU.1*$^{+/+}$*Spi-B*$^{-/-}$ (striped bars), and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ (black bars) animals and stimulated with the indicated mitogens. Data are represented as the mean and standard error of at least four independent experiments. (A) B cells were stimulated with 50 μg/ml $F(ab')_2$ fragment of anti-IgM antibody, 50 μg/ml LPS, or 10 ng/ml PMA and 0.5 μg/ml ionomycin. (B) B cells were stimulated with suboptimal amounts (3 μg/ml) of $F(ab')_2$ fragment of anti-IgM antibody alone or in combination with 10 μg/ml anti-CD40 antibodies or 10 ng/ml recombinant IL-4.

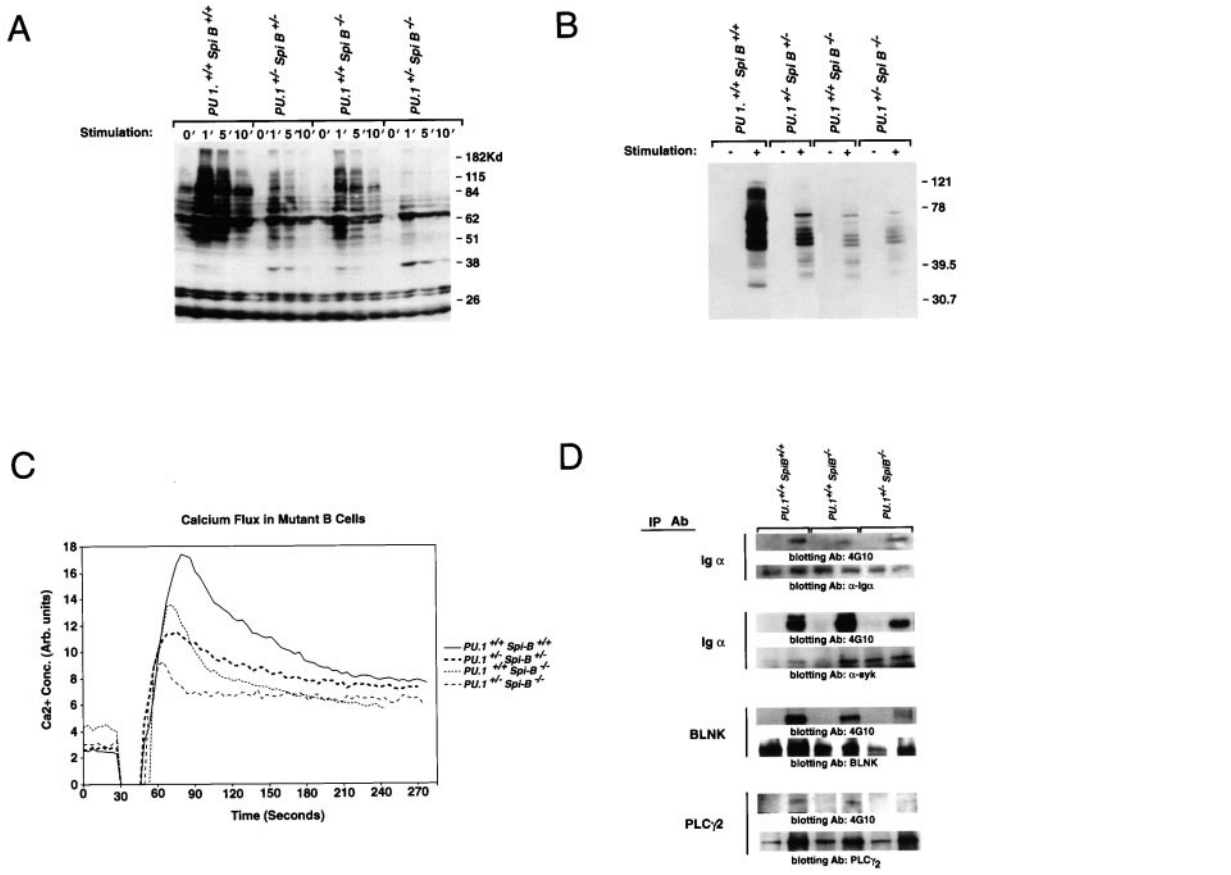

Figure 5. Mutant B Cells Exhibit Reduced BCR Signaling

Protein extracts of splenic B cells purified from mice with the indicated genotypes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the anti-phosphotyrosine mAb 4G10. Prior to lysis, B cells were either mock stimulated with media or stimulated with 50 μg/ml F(ab')$_2$ fragment of anti-IgM antibody for the indicated times (A) or 2.5 mM Na_3VO_4/0.3% H_2O_2 for 1 min (B). (C) Purified splenic B cells were incubated with the Ca^{2+}-sensitive dyes Fluo-3 and Fura-Red. Basal fluorescence ratios of cells at 37°C were measured for 30 s. Anti-IgM antibody was added to 50 μg/ml, and fluorescence changes were measured for an additional 4 min. Results are represented in arbitrary units as changes in fluorescence ratio over time. (D) Lysates from purified splenic B cells were immunoprecipitated with the indicated antibodies. Immunoprecipitates were Western blotted using the anti-phosphotyrosine antibody 4G10. Blots were then stripped and reprobed with the indicated antibodies. The decrease in syk phosphorylation exhibited by *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells compared to wild-type cells was not reproducible, whereas decreases in the phosphorylation of BLNK and PLCγ2 were.

stimuli. To more directly test this, we measured the levels of tyrosine phosphorylation induced by IgM cross-linking. *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, *Spi-B*$^{-/-}$, and *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ B cells exhibited reduced levels of tyrosine phosphorylation compared to wild-type cells, even after 10 min of stimulation (Figure 5A). On the other hand, neither *PU.1*$^{+/-}$ nor *Spi-B*$^{+/-}$ B cells showed a significant tyrosine phosphorylation defect (data not shown). To further characterize the signaling deficiency in mutant B cells, we examined the levels of tyrosine phosphorylation induced by pervanadate/H_20_2 treatment (which inhibits cellular phosphatases). Induction of efficient tyrosine phosphorylation by this compound requires the expression of a functional BCR complex on the cell surface (Wienands et al., 1996), suggesting the need for a preformed BCR signal transducing complex. *Spi-B*$^{-/-}$, *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells exhibited reduced levels of tyrosine phosphorylation in response to pervanadate/H_20_2 treatment compared to wild-type cells (Figure 5B), confirming the lack of intact BCR-mediated signal transduction in mutant B cells. Moreover, these results also indicate that the decreased tyrosine phosphorylation levels evident in mutant cell lysates cannot be explained exclusively by the overactivity or overabundance of a protein tyrosine phosphatase such as SHP-1.

To further analyze BCR-mediated signaling defects in mutant mice, we looked at the ability of mutant B cells to mobilize Ca^{2+} in response to IgM cross-linking using the Ca^{2+}-sensitive dyes Fluo-3 and Fura-Red. Whereas wild-type B cells display a robust response to BCR stimulation, mutant B cells exhibit varying degrees of impairment of Ca^{2+} mobilization with *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells showing the greatest defects (Figure 5C).

Both the reduction in tyrosine phosphorylation and the normal proliferative response to PMA plus ionomycin suggest that mutant B cells are defective in a membrane-proximal step of the BCR signaling pathway. We therefore tested the expression levels of known membrane-proximal protein kinases involved in BCR signal transduction (lyn, fyn, blk, syk, and Btk). Equivalent levels of these kinases were expressed in all genotypes

(data not shown). Furthermore, in vitro kinase assays using enolase as an exogenous substrate demonstrated that the basal kinase activity of syk, lyn, and fyn was similar in wild-type and mutant B lymphocytes (data not shown).

Although all of the membrane-proximal kinases appeared to be expressed at normal levels in wild-type and mutant B cells, it was possible that these kinases were not localized properly to their targets. Recently, an important B cell adaptor protein, BLNK/SLP-65, has been described that appears to mediate the association of the protein kinase syk with several of its putative targets (Fu et al., 1998; Wienands et al., 1998). Western blot analysis detected equivalent levels of BLNK in all genotypes (data not shown). We have also shown that Vav, Igα, and Igβ are present at normal levels in mutant B lymphocytes (data not shown).

To further explore the nature of the signaling defect in mutant lymphocytes, we immunoprecipitated Igα and Igβ from resting and BCR-stimulated B cells and assessed their tyrosine phosphorylation. Igα (Figure 5D) and Igβ (data not shown) are phosphorylated to a similar extent in wild-type, *Spi-B*$^{-/-}$, and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells upon receptor ligation. Moreover, syk was coimmunoprecipitated with Igα/Igβ and phosphorylated to similar extents in wild-type and mutant cells (Figure 5D). Therefore, the first steps of the BCR signaling cascade appear normal in mutant B cells. We next determined if proteins downstream in the BCR signaling cascade, BLNK and PLCγ, were phosphorylated in mutant B cells. Immunoprecipitation of these proteins followed by Western blotting with anti-phosphotyrosine antibodies showed that tyrosine phosphorylation of both proteins is decreased in mutant cells (Figure 5D). Therefore, it appears that syk is recruited to Igα/Igβ in mutant B cells and becomes phosphorylated but fails to couple to downstream targets. We conclude that mutant B cells are deficient in an unknown component of the BCR signaling cascade that is required for the interaction of syk with downstream proteins such as BLNK and PLCγ whose expression is dependent on PU.1 and Spi-B.

Discussion

Reduced Levels of Substrate Tyrosine Phosphorylation upon IgM Cross-Linking in Mutant B Cells

This investigation establishes a novel, unexpected function for the Ets transcription factor PU.1 in BCR signaling. In the absence of Spi-B, it is apparent that PU.1, in a gene dosage-dependent fashion, regulates the expression of one or more genes critical for proper BCR signal transduction. B cells from *Spi-B*$^{-/-}$, *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice exhibit a defective germinal center reaction upon immunization with a T-dependent antigen (DNP-KLH) and proliferate poorly in vitro in response to IgM cross-linking. Since both of these events require signaling through the BCR, we hypothesized that BCR-mediated signal transduction might be defective in mutant B cells. Importantly, PMA and ionomycin (which bypass the BCR and activate downstream signaling pathways) were able to induce normal proliferation in mutant B cells, indicating that it was possible to circumvent the signaling defect present in these cells. We conclude that one or more membrane-proximal signaling events are defective in mutant B cells but that downstream signal transduction pathways are intact and can be activated. In support of this theory, we show that tyrosine phosphorylation initiated by IgM cross-linking is substantially reduced in *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{+/-}$ B cells and nearly absent in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ cells. The genes for the immunoglobulin heavy and light chains contain Ets binding sites in their regulatory regions (Pongubala et al., 1992; Eisenbeis et al., 1993; Nelsen et al., 1993; Shin and Koshland, 1993). A trivial explanation for the reduced BCR signaling observed in mutant B cells is that they express reduced amounts of surface IgM. However, we have shown that both *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells display increased amounts of surface IgM in flow cytometry analyses. Expression of high levels of surface IgM may reflect a compensation for the reduced capacity of the BCR to signal efficiently in mutant B cells.

Lack of tyrosine phosphorylation in lysates of IgM cross-linked cells could be caused by the overexpression or overactivity of a negative regulatory phosphatase (for instance SHP-1). However, both *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells show diminished levels of tyrosine phosphorylation induced by the phosphatase inhibitor pervanadate/H_2O_2 when compared to wild-type B cells. Therefore, decreased phosphorylation in mutant cells cannot be explained by excess phosphatase activity and implies that either kinase activity is deficient or, alternatively, that kinase substrates are not localized properly.

Western analysis using extracts of purified B cells indicates that wild-type and mutant cells express equivalent amounts of lyn, fyn, blk, syk, and Btk. Moreover, mutant B cells also express normal levels of an important membrane-proximal adaptor protein, BLNK/SLP-65. We show that Igα/Igβ is phosphorylated in mutant cells, suggesting that src family kinases are activated upon receptor cross-linking. Moreover, normal amounts of syk are recruited to Igα/Igβ and phosphorylated. Binding of syk to phosphorylated ITAM motifs (Rowley et al., 1995; Shiue et al., 1995) and its phosphorylation (Rowley et al., 1995; Couture et al., 1997) have been shown to activate the enzyme, and it is, therefore, likely that syk is activated in our mutant B cells. Interestingly, however, putative syk target genes BLNK and PLCγ are poorly phosphorylated, suggesting that these substrates may not be properly recruited to syk. Therefore, we propose that *Spi-B*$^{-/-}$, *PU.1*$^{+/-}$*Spi-B*$^{+/-}$, and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells express inadequate levels of an unidentified component(s) of BCR signaling pathways crucial for recruiting BLNK, PLCγ, and other substrates to syk and for normal BCR-mediated events (designated X in Figure 6).

Functional Defects and Increased Rates of Apoptosis in Mutant B Cells

Although all blood cell lineages are present in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice, these animals are characterized by a reduced number of immature and mature B cells in the bone marrow and peripheral lymphoid organs. In contrast, normal numbers of pro- and pre-B cells are observed in the bone marrow. Immature and mature B

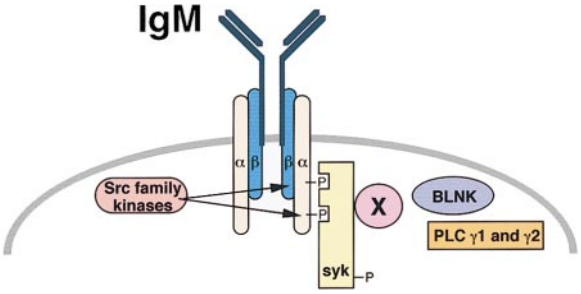

Figure 6. Model of BCR-Mediated Signal Transduction

A simplified model of some of the known components involved in the membrane-proximal steps of BCR signaling. Membrane bound IgM in association with Igα and Igβ constitutes the BCR complex. Src family kinases phosphorylate the ITAM motifs of Igα and Igβ. Syk then binds to the phospho-ITAMs via its SH2 domains and interacts with downstream targets including BLNK and PLCγ. We theorize that *Spi-B*$^{-/-}$ and *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells lack expression of some critical component X, which is required for coupling downstream proteins to syk.

cell populations in the spleens of *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice exhibit increased binding of Annexin V, indicating that they are undergoing increased rates of apoptosis. Increased apoptosis within these populations likely accounts for the reduced number of peripheral B cells that characterizes *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice. Poor BCR-induced tyrosine phosphorylation is observed in antigen-desensitized and anergic B cells (Cooke et al., 1994; Vilen et al., 1997), but unlike *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells, they express reduced levels of surface IgM. Normal peripheral B cells appear to require a surface Ig and presumably a low level of BCR signaling to survive (Lam et al., 1997), and anergic B cells, which exhibit reduced BCR signaling, undergo increased rates of apoptosis. *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells exhibit a severe defect in substrate tyrosine phosphorylation upon BCR stimulation despite expressing increased levels of surface IgM. A reasonable explanation for the increased rates of apoptosis among *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ immature and mature B cells is that they receive insufficient BCR-dependent signals to permit survival.

It could be hypothesized that the lack of a strong positive signal from the BCR might interfere with negative selection and result in the production of increased numbers of autoreactive B cells in mutant mice. At present, we have no evidence for the production of autoreactive cells in *Spi-B*$^{-/-}$ nor in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice. In contrast, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice show a decreased number of peripheral B cells. A previous study has demonstrated the requirement for a positive signal through the BCR complex to allow maturation of B cells into the peripheral pool (Torres et al., 1996). It may be that, in addition to the increased apoptosis observed in *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells, mutant cells also fail to mature properly due to lack of a positive selection.

Normal B Lymphopoiesis in Mutant Mice

Given the extremely inefficient tyrosine phosphorylation evident in Figure 5A, it is surprising that *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B cells undergo lymphopoiesis apparently normally. Proliferation and differentiation of B lineage precursors in the bone marrow requires signal transduction through a pre-BCR complex consisting of the Ig heavy chain in association with surrogate light chains and the Igα and Igβ molecules. The pre-BCR appears to signal through at least some of the same protein components as the BCR (Cheng et al., 1995; Turner et al., 1995; Gong and Nussenzweig, 1996). Other signaling components of the BCR pathway are presumed to play a role in pre-BCR signaling. However, our data support the idea that the signaling components of the pre-BCR are not identical to those of the BCR.

In summary, we have shown that both PU.1 and Spi-B are critical for normal BCR signal transduction. Weak BCR signaling is associated with poor B cell function, increased apoptosis, and reduced numbers of immature and mature B cells. B cells from mutant mice fail to initiate proper tyrosine phosphorylation upon BCR ligation although normal levels of all known proximal BCR signaling proteins are detected. Although syk is recruited to the BCR complex and becomes phosphorylated, its putative targets display reduced phosphorylation. We propose that PU.1 and Spi-B together regulate an unknown component(s) of the BCR signaling cascade required for coupling syk to its substrates. As such, *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ B lymphocytes represent an ideal model system to identify additional proximal molecules essential for these events.

Experimental Procedures

Cell Counts and Flow Cytometry Analysis

Single cell suspensions were prepared from the indicated tissues and lysed with ammonium chloride buffer. For flow cytometry analysis, each sample was stained with FITC-labeled monoclonal antibody (mAb) (IgM or CD43; Pharmingen), PE-labeled mAb (B220, IgD^{a}, or IgD^{b}; Pharmingen), and/or biotin-conjugated mAb (IgM, S7, HSA, or BP-1; Pharmingen) followed by cychrome-labeled streptavidin (Pharmingen) as previously reported (Su et al., 1997). For analysis of apoptotic B cells in bone marrow and spleen, a single cell suspension was stained first with PE-labeled IgDa, PE-labeled IgDb, and biotin-conjugated IgM followed by cychrome-labeled streptavidin. Cells were washed in 1X binding buffer supplied in the Apoptosis Detection Kit (R & D Systems) and then incubated with FITC-labeled Annexin V. Labeled cells were analyzed by the LYSIS II program or the CellQuest program of a FACScan instrument (Becton Dickinson) and quantitated by CellQuest or WINmidi software. Ten thousand events were collected per dot plot.

Antigenic Challenges and Immunohistochemistry

Mice were immunized with DNP-KLH, and immunohistochemistry was performed to determine the number of B220^{+}/TUNEL^{+} cells in sections of spleens as previously described (Su et al., 1997).

Cell Purification and Proliferation

B cells were purified from mouse spleens as previously described (Su et al., 1997). B cell purity as assayed by flow cytometry with a B220 mAb was approximately 95% for wild-type, Spi-B$^{-/-}$, and PU.1$^{+/-}$Spi-B$^{+/-}$ animals and 85% for PU.1$^{+/-}$Spi-B$^{-/-}$ (the contaminating cells were largely TER119^{+} erythroid precursors). In vitro proliferation assays were performed as previously described (Su et al., 1997). Anti-IgM antibody (50 or 3 μg/ml, affinity pure F(ab′)$_2$ fragment, goat anti-mouse IgM, μ chain specific; Jackson ImmunoResearch Laboratories), LPS (50 μg/ml), PMA (10 ng/ml) plus ionomycin (0.5 μg/ml), anti-CD40 antibody (10 μg/ml, purified rat anti-mouse CD40; Pharmingen), and IL-4 (10 ng/ml, recombinant mouse IL-4; Pharmingen) were given as soluble stimulants. Cells were pulsed with [^{3}H]-thymidine at 1 mCi/ml after 48 hr and counted 16 hr after thymidine treatment.

Cell Stimulation, Western Blotting, and Immunoprecipitations

B cells purified from mouse spleens were resuspended at 4×10^7 cells/ml in 1× Dulbecco's phosphate-buffered saline (PBS) (GIBCO-BRL) and incubated at 37°C for 10 min prior to stimulation. Cells were then stimulated for 1 min with either media alone, anti-IgM antibody (50 μg/ml, affinity purified F(ab')2 fragment, goat anti-mouse IgM [μ chain specific] Jackson ImmunoResearch Laboratories), or a sodium pervanadate solution (0.3% H_2O_2 and 2.5 mM Na_3VO_4). Cells were lysed by adding an equal volume of 2× NETN Buffer (40 mM Tris HCl [pH 8.0], 275 mM NaCl, 40% glycerol, 2% NP-40, 4 mM EDTA [pH 8.0], 2 mM Na_3VO_4, 20 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin, and 20 μg/ml leupeptin) and then incubated on ice for 20 min and centrifuged to remove cellular debris.

For immunoprecipitations, lysates of 5×10^6 cells prepared as described above were incubated with antibody (3 μg rabbit anti-Igα antiserum purified by Protein A chromatography, 2 μg anti-PLCγ2 [Santa Cruz Biotechnology], or 5 μl of anti-BLNK antiserum [a kind gift of Dr. Andrew Chan]) followed by the addition of Protein A sepharose (Sigma). Immunoprecipitates were collected by centrifugation and washed three times with ice-cold 1× NETN buffer. After a final wash, beads were resuspended in 2× SDS PAGE gel loading buffer and boiled.

Samples were boiled and applied to 8%–10% SDS polyacrylamide gels for electrophoresis. Proteins were transferred to nitrocellulose as described (Sambrook et al., 1989) and membranes were blocked in 5% nonfat dry milk in TBST (10 mM Tris HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) or in 3% bovine serum albumin (BSA) in TBST. Primary antibodies (4G10 anti-phosphotyrosine monoclonal [Upstate Biotechnology], anti-Igα polyclonal, anti-PLCγ2 polyclonal, and anti-BLNK polyclonal [described above], or anti-syk polyclonal [a kind gift of Dr. J. Cambier]) were diluted to appropriate concentrations and incubated with the membranes overnight at 4°C. Horseradish peroxidase-labeled goat anti-rabbit or goat anti-mouse secondary antibody (GIBCO-BRL) was incubated at 1:5000 dilution in 5% milk/TBST or 3% BSA/TBST. The membranes were then washed and developed with a LumiGLO Chemiluminescent Detection Kit (Kirkegaard and Perry Laboratories [KPL]) to visualize the signal.

Measurement of Intracellular Calcium Concentration

Intracellular calcium elevation was measured as described by Novak and Rabinovitch (1994). In brief, purified B cells (10^7 cells/ml) were incubated at 37°C for 45 min with Fluo-3 (Molecular Probes) and Fura-Red (Molecular Probes) in RPMI-1640 supplemented with 5% FBS. Cells were washed and held on ice until stimulation. Immediately prior to stimulation, cells were warmed to 37°C for 2 min, and basal Ca^{2+} concentration was measured for 30 s. Stimulation through the BCR was achieved with αIgM (Jackson Immunoresearch) at 50 μg/ml. The changes in fluorescence were determined for an additional 4 min after αIgM treatment.

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